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(54) **Beta-hydroxybutyrate polymers.**

(57) High molecular weight copolymers containing β-hydroxybutyric acid residue, i.e. units of the formula

$$- O.CH(CH_3).CH_2.CO -$$

and up to 50 mole % of residues of other hydroxy acids, viz units of the formula

$$- O.CR^1R^2.(CR^3R^4)_n.CO -$$

where n is 0 or 1 and, if n = 1 and R^2, R^3, and R^4 = H, R^2 is not methyl.

The copolymers are made microbiologically: for part of the cultivation the micro-organism is under conditions of limitation of a nutrient, e.g. nitrogen source, required for growth but n t polyester accumulation. For at least part of this period of growth limitation the substrate is an acid or a salt thereof that tives the comonomer units. Propi nic acid, which gives p lymers where n = 1, R^2=R^3=R^4=H and R^1 = C_2H_5, is the preferred acid.

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β-hydroxy butyrate polymers

This invention relates to poly(β-hydroxybutyric acid), hereinafter referred to as PHB.

PHB is accumulated by various micro-organisms, principally bacteria, as an energy reserve material as granules within the microbial cells.

PHB extracted from such cells is a thermoplastic polyester of the repeat structure

$$-O.CH(CH_3).CH_2.CO-$$

that rapidly crystallises to a relatively high level e.g. of the order of 70% or more. This crystallisation behaviour is often disadvantageous when the polymer is to be used as, for example, a moulding material.

We have found that the crystallisation of PHB can be modified by incorporation of units of a dissimilar monomer into the polymer chain.

In the following description of the metabolic pathways leading to the polymer synthesis, the following abbreviations are employed:

CoASH is unesterified Coenzyme A. So $CH_3.CO.S.CoA$ is the acetyl thioester of Coenzyme A and is more commonly termed acetyl CoA.

NADP is nicotinamide adenine dinucleotide in the oxidised state. $NADPH_2$ is reduced NADP.

It is believed that, in the biosynthesis of PHB by a micro- rganism the first step is the synthesis of acetyl CoA.

This can be formed, for example, from Coenzyme A and acetate, or by the decarboxylation of pyruvate, which is a product of the glycolysis of carbohydrates, or which can be formed by decarboxylation of oxaloacetate, the latter being a member of the tricarboxylic acid, TCA, cycle, otherwise known as the Krebs cycle.

Thus with acetate as the source of acetyl CoA, the PHB is produced by a metabolic pathway involving the reactions:

1. $CH_3.CO.O^- + CoA.SH \xrightarrow{\text{thiokinase}} CH_3.CO.S.CoA + OH^-$

2. $2CH_3.CO.S.CoA \xrightarrow{\beta\text{-ketothiolase}} CH_3.CO.CH_2.CO.S.CoA + CoA.SH$

3. $CH_3.CO.CH_2.CO.S.CoA + NADPH_2 \xrightarrow{\text{reductase}} CH_3.CHOH.CH_2.CO.S.CoA + NADP$
   β-hydroxybutyryl CoA

4. $CH_3.CHOH.CH_2.CO.S.CoA + (-O.CH(CH_3).CH_2.CO-)_{n-1} \xrightarrow{\text{polymerase}} (-O.CH(CH_3).CH_2.CO-)_n + CoA.SH$

where $(-O.CH(CH_3).CH_2.CO-)_{n-1}$ is a PHB molecule containing n-1 repeat units. Thus reaction 4 adds a $-O.CH(CH_3).CH_2.CO-$ unit to the polymer chain.

We have found that a minor proportion of comonomer units may be introduced into the polymer chain by cultivation of the micro-organism under certain conditions in the presence of certain organic acids. To be of any practical use as plastics materials, the polymers should have a weight average molecular weight, (Mw), above 10,000 e.g. as measured by gel permeation chromatography.

Accordingly we provide copolymers having a weight average molecular weight above 10,000 and containing 99.9 to 50 mole % of repeat units

I $- O.CH(CH_3).CH_2.CO -$

and 0.1 to 50 mole % of repeat units

$$-O.CR^1R^2.(CR^3R^4)_n.CO-$$

where n is 0 or 1 and R^1, R^2, R^3 and R^4 are each selected from hydrocarbon radicals, such as alkyl, aralkyl, aryl, or alkaryl radicals; halo- and hydroxy- substituted hydrocarbon radicals; hydroxy radicals; halogen atoms; and hydrogen atoms, provided that, where n is 1, if R^2, R^3 and R^4 are each hydrogen atoms, R^1 is not methyl.

Preferably the groups R^1, R^2, R^3 and R^4 each contain less than 4 carbon atoms. Generally at least one of the groups R^1, R^2, R^3 and R^4 is hydrogen.

Such copolymers may be produced since the enzymes involved have a degree of non-specificity.

Thus the enzyme, thiokinase, involved in reaction 1 has a broad specificity: it will attach Coenzyme A to a variety of other carboxylate groups according to the general reaction

1a. $R.CO.O^- + CoA.SH \xrightarrow{\text{thiokinase}} R.CO.S.CoA + OH^-$

e.g. $CH_3.CH_2.CO.O^- + CoA.SH \longrightarrow CH_3.CH_2.CO.S.CoA + OH^-$

propionyl CoA

Reaction 2, involving the enzyme β-ketothiolase, can be rewritten

2. $CH_3.CO.S.CoA + CH_3.CO.S.CoA \longrightarrow CH_3.CO.CH_2.CO.S.CoA + CoA.SH.$

This reaction appears to be partially specific in that one reactant must be acetyl CoA. The general reaction is thus

2a. $R.CO.S.CoA + CH_3.CO.S.CoA \longrightarrow R.CO.CH_2.CO.S.CoA + CoA.SH$

Likewise the specificity of the reductase enzyme involved in reaction 3 is degenerate and will reduce certain fatty acyl thioesters of the general formula $R.CO.CH_2.CO.S.CoA$ as follows:

3a. $R.CO.CH_2.CO.S.CoA + NADPH_2 \longrightarrow R.CHOH.CH_2.CO.S.CoA + NADP$

The polymerase enzyme of reaction 4 is not absolutely specific. The general reaction can be written:

4a. $R.CHOH.CH_2.CO.S.CoA + (-O.CHR^1.CH_2.CO-)_n \longrightarrow (-O.CHR^1.CH_2.CO-)_n-O.CHR.CH_2CO- + CoA.SH$

in which R and R^1 may differ.

This route therefore gives rise to polymers containing units of the formula

$$- O.CHR^1.CH_2.CO -$$

i.e. units

$$- O.CR^1 R^2.CR^3 R^4.CO -$$

where R^2, R^3, and R^4 are hydrogen. Hence copolymers can be produced if, in some of the repeat units, R^1 is not methyl.

The β-hydroxyl thioester, e.g. $R.CHOH.CH_2.CO.S.CoA$, reactant of reaction 4a can also be made, in certain cases, by the reactions catalysed by the non-specific fatty acid metabolism enzyme enoylhydratase:

5a. $R^1 R^2 C = CR^3.CO.O^- + H_2O \xrightarrow{\text{enoylhydratase}} R^1R^2 C(OH).CHR^3.CO.O^-$

5b. $R^1 R^2 C(OH).CHR^3.CO.O^- + CoASH \longrightarrow R^1 R^2 C(OH).CHR^3.CO.S.CoA + OH^-$

(The reactions 5a, 5b may in fact be reversed, i.e. the hydration of the carbon-carbon double bond may occur after thioesterification). R^1, R^2 and R^3 are not necessarily hydrogen.

Hence by the use of reactions 5a, 5b and 4a, units of the formula

$$- O.CR^1R^2.CHR^3.CO -$$

i.e. units

$$- O.CR^1R^2.CR^3R^4.CO -$$

where R^4 is hydrogen, can b introduced into the polymer chain. Hence copolymers may be produced provided that in some of the repeat units R^1 is not methyl if R^2 and R^3 are hydrogen.

Also the polymerase enzyme of reaction 4a may be non-specific so that it can utilise some reactants having the hydroxyl group in the α-position, for example reactants of the type

$$R^1R^2C(OH).CO.S.CoA$$

which introduces units of the formula

$$-O.CR^1R^2.CO-$$

i.e. units of the formula

$$-O.CR^1R^2.(CR^3R^4)_n.CO-$$

where n is 0, into the polymer chain.

The polymerase enzyme may also, in some cases, utilise β-hydroxy reactants of the formula

$$R^1R^2C(OH).CR^3R^4.CO.S.CoA$$

These reactants can be made from the corresponding β-hydroxy acids by reaction 1a, i.e.

$$R^1R^2C(OH).CR^3R^4.CO.S.CoA \xrightarrow{\text{thiokinase}} R^1R^2C(OH).CR^3R^4.CO.S.CoA + OH^-$$

e.g. β-hydroxybutyric acid which gives β-hydroxybutyryl CoA and pivalic acid which gives pivalyl CoA

$$CH_2(OH).C(CH_3)_2.COO^- + CoA.SH \longrightarrow CH_2(OH).C(CH_3).CO.S.CoA + OH^-$$

Such reactants can thus introduce units of the formula

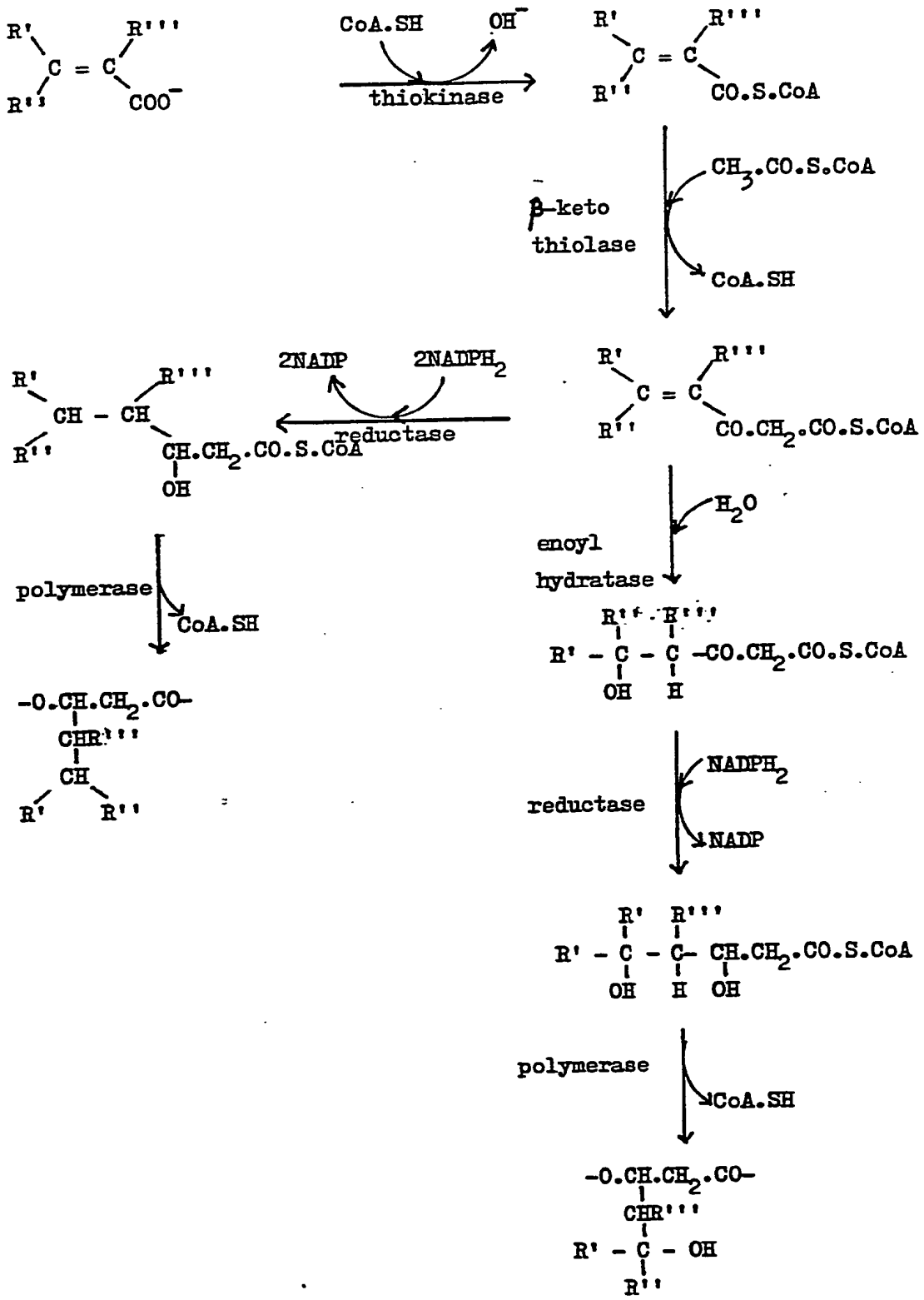
$$-O.CR^1R^2.CR^3R^4.CO-$$

into the polymer chain, and so copolymers can be produced provided that in some of the repeat units R^1 is not methyl if R^2, R^3 and R^4 are hydrogen.

With some unsaturated acids, instead of reactions 5a, 5b occuring it is also possible that the polymer synthesis proceeds via a route involving reactions 2a and 3a, as well as hydration of the carbon-carbon double bond by a reaction such as 5a or reduction of the carbon-carbon double bond, e.g. by a reaction

6. $$CR'R'' = CR'''.CO.O^- + NADPH_2 \xrightarrow{\text{reductase}} R'R''CH.CHR'''.CO.O^- + NADP$$

Thus one possible sequence is as follows



These routes thus give copolymers containing the units

$$-O.CHR^1.CH_2.CO-$$

i.e.

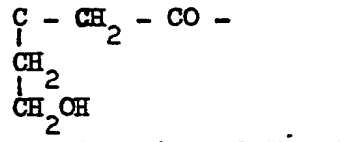
$$-O.CR^1R^2.CR^3R^4.CO-$$

units where R^2,R^3 and R^4 are hydrogen and R^1 is

$$-CHR'''.CHR'R''$$ and/or $$-CHR'''.C(OH)R'R''$$

The proportion of repeat units II in the copolymer is between 0.1 and 50, particularly 1 to 40, mole percent of the total repeat units in the copolymer. In some cases the polymer produced by the micro-organism may be a blend of a homopolymer of repeat units I with a copolymer containing repeat units I and II. In this case the overall proportion of repeat units II in the polymer is between 0.1 and 50 mole percent of the total repeat units. Preferably the proportion of repeat units in the polymer is between 3 and 30 mole %.

We have found that, instead of following the course of the above reactions, in some cases the micro-organism will perform elimination reactions in addition to, or instead of, some of the reactions outlined above, giving polymers containing β-hydroxyvaleric acid units, and/or 3,5 dihydroxypentanoic acid units linked into the polymer chain via the hydroxyl group in the 3 position, i.e.



units. The copolymers thus contain units of the formula

$$-O.CHR^1.CH_2.CO-$$

where R^1 is ethyl or 2-hydroxyethyl.

Copolymers in which n = 1, R^1 is ethyl and R^2,R^3 and R^4 are hydrogen are preferred.

Certain polymers containing β-hydroxybutyric acid units, i.e.

$$-O.CH(CH_3).CH_2.CO-$$

units, and other units have been described in the literature.

Thus polymers exhibiting an infra-red band said to be indicative of ethylenic unsaturation are described by Davis in "Applied Microbiology" 12 (1964) pages 301 - 304. These polymers

which are said by Davis to be copolymers containing β-hydroxybutyric acid units and 3-hydroxy-2-butenoic acid units, i.e. units of the formula

$$- O.C(CH_3) = CH.CO -$$

were prepared by cultivating Nocardia on n-butane.

Also Wallen et al describe in "Environmental Science and Technology" 6 (1972) pages 161 - 164 and 8 (1974) pages 576 - 579 a polymer melting at 97 - 100°C (after repeated washing) isolated from activated sludges and containing β-hydroxybutyric acid units and β-hydroxyvaleric acid units, i.e.

$$- O.CH(C_2H_5).CH_2.CO -$$

units in the ratio of 1:5. The polymer thus contains only about 17% of β-hydroxybutyric acid units. Marchessault et al report in "IUPAC Macro Florence 1980 International Symposium on Macromoles Preprints" 2 (1980) pages 272 - 275 a study of this polymer and confirmed that it contained mainly β-hydroxyvaleric acid units.

United States Patent Specification 3275610 describes the microbiological production of polyesters by cultivating certain micro-organisms, especially Nocardia salmonicolor, on carboxylic acids containing 4 carbon atoms. In Examples 2 and 3, where the acids were 3-butenoic and α-hydroxybutyric acids respectively, the polymers appear, from the quoted melting points of the order of 178 - 184°C, to be poly(β-hydroxybutyric acid). In Example 1 however, wherein 2-methyl acrylic acid, i.e. methacrylic acid, was employed the polymer produced is unidentified but is described as having a melting point of 215 - 220°C and as being soluble in methyl ethyl ketone. In contrast thereto, copolymers in accordance with the present invention, containing predominantly β-hydroxybutyric acid residues, have melting points below 180°C and are insoluble in cold methyl ethyl ketone.

When PHB-accumulating micro-organisms are aerobically cultured on a suitable substrate, i.e. a source of energy and carbon, they reproduce until one or more of the essential requirements for reproduction is exhausted. This r production of the micro-organism is hereinafter referred to as growth. Upon

exhaustion of an essential growth requirement, further growth occurs only to a very limited extent, if at all, but, providing the substrate is not exhausted, PHB may be accumulated by the micro-organism.

With some micro-organisms, even in the absence of a PHB-inducing constraint such as a limitation on one or more of the essential growth requirements, PHB may also be accumulated while growth of the micro-organism is taking place: however the amount of PHB so accumulated is generally small and typically is less than about 10% by weight of the cells produced. Thus when grown in batch culture, the micro-organism will grow, with little or no PHB accumulation until one or more of the essential requirements for growth becomes exhausted, and then the micro-organism synthesises PHB.

We have found that in order to produce the copolymers it is generally necessary to use the acid that is to give rise to the comonomer units as at least part of the substrate during cultivation of the micro-organism under conditions wherein the amount of one of more of the essential requirements for growth, but not PHB accumulation, is limited. Where there is no such restriction of an essential requirement for growth, the acid will generally be metabolised by the micro-organism by other pathways leading to e.g. acetyl CoA or to a member of the TCA cycle, and so copolymers will not be produced. Thus, as an example, propionic acid can be metabolised by micro-organisms, in the absence of any growth limitation, via propionyl CoA, with the incorporation of carbon dioxide to methyl malonyl CoA, and thence to succinate, a member of the TCA cycle.

We therefore provide a process for the production of a thermoplastic polyester wherein a micro-organism that is capable of accumulating a polyester is cultivated in an aqueous medium on a water soluble assimilable carbon containing substrate with at least part of the cultivation being conducted under conditions of limitation f one or more of the essential requirements for microbial growth, but not poly ster accumulation,

characterised in that, during at least part of the period when the cultivation is so limited, the substrate comprises an organic acid, or salt thereof, that is metabolisable by said micro-organism under said limitation conditions, to a polyester other than one composed solely of - O.CH(CH_3).CH_2.CO - repeat units.

In this regard it is noted that, in United States Patent 3275610 mentioned above, the amounts of cells produced were such that no growth limitation were imposed.

In addition to the substrate and oxygen (which is generally supplied by injecting air into the aqueous medium in the fermenter), various nutrient salts are required to enable the micro-organism to grow. Thus sources of the following elements in assimilable form, normally as water soluble salts, are generally required: nitrogen, phosphorus, sulphur, potassium, sodium, magnesium, calcium, and iron, together with traces of elements such as manganese, zinc and copper. While it may be possible to induce polyester accumulation by restricting the supply of oxygen to the fermenter, it is preferred to restrict the amount of one or more of the nutrient salts. The most practical elements to limit are nitrogen, phosphorus, or, less preferably, magnesium, sulphur or potassium. Of these it is most preferred to restrict the amount of nitrogen (which is conveniently supplied as an ammonium salt). The amount of assimilable nitrogen required is about 8 - 15% by weight of the desired weight of cells less accumulated polyester.

The fermentation is preferably conducted so that the dry weight of the polyester-containing cells is at least 5 g per litre of aqueous medium. Hence if, for example, it is desired to produce 10 g per litre of PHB-containing cells having a PHB content of 40% by weight, the amount of the essential nutrient fed to the fermenter that is used to limit the amount of cell growth must be that required to support the growth of 6 g per litre of cells containing no PHB: thus, if nitrogen is employed as the growth limiting growth limiting nutrient, since the nitrogen content of PHB free bacterial cells is about 8 - 15% by weight,

the amount of assimilable nitrogen required would be between about 0.5 and 0.9 g per litre, e.g. about 0.6 to 1.2 g of ammonium ions per litre.

The fermentation may be conducted under the conditions e.g. pH, temperature, and degree of aeration (unless oxygen is utilised as the limiting nutrient) conventionally used for the micro-organism. Likewise the amounts of nutrient salts (other than the growth limiting nutrient whose amount may be determined following the considerations outlined hereinbefore) employed may be those normally used for growth of the micro-organism.

The micro-organism is preferably grown to a certain desired weight by cultivation in the presence of sufficient of the nutrient required for growth that is to be restricted in the polymer accumulation stage on a readily metabolisable substrate, such as a carbohydrate, and then cultivated under conditions of growth requirement restriction to cause the polymer accumulation. In some cases the substrate for at least part, and in some cases all, of the growth stage may be the acid that gives rise to the copolymer repeat units II in the polymer accumulation stage.

The fermentation may be performed as a batch fermentation in which case polymer accumulation will occur as the amount of the nutrient that is required for growth but not polymer accumulation becomes depleted, i.e. exhausted. Alternatively the fermentation may be conducted as a continuous process wherein aqueous medium containing the bacterial cells is removed, continuously or intermittently, from the fermentation vessel at a rate corresponding to the rate of addition of fresh aqueous medium and substrate thereto. It is preferred that the amount of the nutrient that is restricted that is fed to the fermentation vessel is such that the aqueous medium removed from the vessel contains little or none of that nutrient, and the aqueous medium removed from the vessel is then fed to a second fermentation vessel, operated either in batch or, preferably, continu us fashion wher in polymer accumulation is caused to take place by continuing the aerobic cultivation with the addition f a fresh

quantity of substrate comprising the copolymer producing acid. While additional quantities of substrate and nutrient salts may be added in this further fermentation step, since further growth is generally not desired, little or no further quantity of the nutrient utilised to limit growth should be added. It will however be appreciated that the aqueous medium fed to the further fermenter or fermenters from the first fermenter may contain some residual quantity of the limiting nutrient and/or the addition of a further small quantity thereof may be desirable for efficient operation.

In either a batch process, or a continuous process as described above, the acid used to provide the copolymer repeat units II is used as part, or all, of the substrate during the polymer accumulation stage occurring upon exhaustion of the nutrient required for growth. The acid may be used in admixture with a substrate, e.g. a carbohydrate, that will give repeat units I, or may be the sole substrate: in the latter case sufficient of the acid will normally be metabolised by other pathways to acetyl CoA to provide the repeat units I and any acetyl CoA required to produce the repeat units II, i.e. if a pathway involving reaction 2a, is employed. However, when the acid is the sole substrate, the yield of polymer is often low.

The acid giving repeat units II may be present for only part of the polymer accumulation stage: for the rest of the polymer accumulation stage, which may occur before and/or after the part of the polymer accumulation stage wherein the acid is present, a substrate giving only repeat units I may be the sole substrate.

In some cases it may be possible to prevent the "normal" metabolisation of the acid, i.e. to acetyl CoA, by blocking enzymes required for that pathway and/or by using micro-organisms that lack the ability to synthesise the necessary enzymes. However in order to obtain substantial yields of polymer a peri d of cultivation under conditions of limitation, and preferably depletion, of a nutrient required for growth is generally desirable.

The fermentation is preferably conducted so that the amount of accumulated polyester comprises about 50 to 80% by weight of the bacterial cells.

Acids that can be used to produce copolymers should be those that do not give rise only to repeat units I, when the cultivation is in the growth limited state. Unsuitable acids therefore include acetic and β-hydroxybutyric acids, members of the TCA cycle, and acids giving only acetyl CoA and/or a member of the TCA cycle when the cultivation is in the growth limited state. Thus unsuitable acids also include phosphoglyceric, pyruvic, citric, isocitric, α-ketoglutaric, succinic, fumaric, maleic, malic, oxalacetic, oxalosuccinic, aconitic, and methyl malonic, acids. Amino acids are likewise unsuitable. Butyric acid, which undergoes β-oxidation to β-hydroxybutyric acid, is likewise unsuitable. Formic acid does not give copolymers because the enzyme thiokinase does not add coenzyme A to formate.

Suitable acids include propionic, isobutyric, halo- or hydroxy- substituted derivatives of these and butyric acid, e.g. 3-chloropropionic, 3-hydroxypropionic, α-hydroxybutyric (but not β-hydroxybutyric), pivalic, haloacetic, phenyl acetic, and benzoic acids, and unsaturated acids, or halo substituted derivatives thereof such as acrylic, methacrylic (i.e. 2-methyl acrylic), 3,3-dimethylacrylic, 2,3-dimethyl acrylic, 3-chloropropenoic, and 2-chloropropenoic acids.

The substrate should be water soluble and so the acid may be added as such if water soluble or as a water soluble salt, e.g. an alkali metal, salt thereof.

As mentioned here in before, in some cases the micro-organism may perform further reactions on the acid: thus isobutyric acid would be expected to give repeat units II in which n is 1, $R^2 = R^3 = R^4 = H$, and R^1 is isopropyl. In fact repeat units of the type II wherein n is 1, $R^2 = R^3 = R^4 = H$, and R^1 is ethyl are found indicating that the micro-organism here substitutes hydrogen for a methyl group during the metabolic pathway to the copolymer.

Predicted values for n, R^1, R^2, R^3 and R^4 in repeat units II for various acids is as follows:

acid	R^1	R^2	R^3	R^4	n
propionic	ethyl*	H	H	H	1
isobutyric	isopropyl*	H	H	H	1
3-chloropropionic	2-chloroethyl***	H	H	H	1
3-hydroxy propionic	H or 2-hydroxy ethyl	H	H	H	1
acrylic	H or 2-hydroxy ethyl**	H	H	H	1
3,3-dimethyl acrylic	methyl	methyl	H	H	1
	or 2-methyl propyl*	H	H	H	1
	or 2-hydroxy 2-methyl propyl	H	H	H	1
2,3-dimethyl acrylic	methyl	H	methyl	H	1
	or 1-methyl-2-hydroxy propyl	H	H	H	1
	or 1-methyl propyl*	H	H	H	1
2-methyl acrylic	H	H	methyl	H	1
	or isopropyl*	H	H	H	1
	or 1-methyl-2-hydroxy ethyl	H	H	H	1
3-chloropropenoic	Cl or 2-chloroethyl	H	H	H	1
	or 2-chloro-2-hydroxy ethyl	H	H	H	1
2-chloropropenoic	H	H	Cl	H	1
	or 1-chloroethyl	H	H	H	1
	or 1-chloro-2-hydroxy ethyl	H	H	H	1
chloroacetic	chloromethyl****	H	H	H	1
α-hydroxybutyric	ethyl	H	-	-	0
pivalic	H	H	methyl	methyl	1

* thyl found

** 2-hydroxy ethyl found

** thyl and 2-hydroxy ethyl found

**** m thyl found

Micro-organisms that may be used include any poly (β-hydroxybutyric acid) accumulating micro-organisms that are capable of assimilating the acid (or salt thereof) from which it is desired to produce the copolymers. The bacteria Alcaligenes eutrophus (previously known as Hydrogenomonas eutropha) species, e.g. strain H 16 widely employed in academic studies of this species, see e.g. J General Microbiology (1979) 115 pages 185 - 192, and which is available as ATCC strain 17699, and mutants of strain H 16 such as mutants 11/7B, S301/C5, S501/C29 and S501/C41, which have been deposited, on 18 August 1980 with the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen, Scotland, under NCIB Nos. 11600, 11599, 11597 and 11598 respectively, are particularly suitable. The ATCC number refers to the number designated by the American Type Culture Collection, 12301 Park Lawn Drive, Rockville, Maryland 20852, U.S.A. As mentioned hereinbefore a carbohydrate is preferably used as the substrate during the growth stage. While Alcaligenes eutrophus strain H 16 (ATCC 17699) will not utilise glucose, certain mutants thereof, e.g. the aforesaid mutants 11/7B, S301/C5, S501/C29 and S501/C41 can utilise glucose. Carbohydrates, particularly glucose, are the preferred substrates for the growth stage in view of the cost and the fact that the micro-organisms can grow efficiently thereon.

The polyester is produced as granules inside the micro-organism cells. While the cells containing the polyester may themselves be used as a moulding material, for example as described in USP 3,107,172, it is generally desirable to separate the polyester from the bacterial cells. This may be accomplished by subjecting the cells to a cell breakage step followed by extraction of the polyester with a suitable solvent. Examples of suitable extraction processes are described in our European Patent Application 15123.

As mentioned hereinbefore the copolymers should have a weight average molecular weight (Mw) above 10,000 as measured by gel permeation chromatography, if they are to be of any practical

use. Preferably Mw is above 50,000, more preferably above 100,000 and in particular above 200,000.

The copolymers invariably have the D-configuration and xhibit melting points below that of the β-hydroxybutyric acid homopolymer.

The copolymers are of particular utility in the preparation of melt-fibricated articles, where their reduced crystallinity compared to β-hydroxybutyric acid homopolymer is often advantageous.

Of particular interest is the use of small amounts of the copolymers as high molecular weight processing aids for vinyl chloride polymers. For this application the amount of copolymer is preferably 0.5 to 10% by weight of the vinyl chloride polymer. For the best results in this application, we have found that the copolymer should be random: to obtain random copolymers, the acid used to produce the comonomer units II is preferably the sole substrate present, at least throughout the period of cultivation of the micro-organism under the conditions of growth requirement limitation.

Copolymers are also of particular utility in the production of film by melt extrusion, preferably followed by rolling, e.g. passage through one or more pairs of rolls, to reduce the film thickness and induce some orientation, at a temperature between the glass transition temperature (Tg) and the melting point of the polymer.

The invention is illustrated by the following examples.

EXAMPLE 1

In the normal metabolism of propionate, the latter is converted, as described hereinbefore, to succinate which can give rise to acetyl CoA by oxidation in the TCA cycle to oxaloacetic acid followed by decarboxylation. In the decarboxylation of oxal acetic acid both terminal acid groups are removed as carbon dioxide. Hence if propionate having the carbon atom of the carboxyl group radio labelled, i.e. 1.- ^{14}C- propionate, is supplied to the cells conversion to acetyl CoA will result in loss

of the radioactivity as $^{14}CO_2$. Any incorporation of ^{14}C into the polymer must result from conversion of propionyl CoA into β-hydroxyvaleryl CoA and subsequent polymerisation.

Alcaligenes eutrophus mutant NCIB 11599 was grown by aerobic cultivation in a batch fermenter employing an aqueous medium A which contained sufficient assimilable nitrogen to support a biomass free of accumulated polyester of 3.5 g l^{-1} and glucose as the substrate. Medium A had the composition, per litre of deionised water:

$(NH_4)_2 SO_4$	2 g
$MgSO_4.7H_2O$	0.8 g
K_2SO_4	0.45 g
H_3PO_4 (1.1 M)	12 ml
$FeSO_4.7H_2O$	15 mg
Trace element solution	24 ml

The trace element solution had the following composition, per litre of de-ionised water

$CuSO_4.5H_2O$	0.02 g
$ZnSO_4.6H_2O$	0.1 g
$MnSO_4.4H_2O$	0.1 g
$CaCl_2.2H_2O$	2.6 g

When the biomass concentration reached 4.5 g l^{-1}, i.e. after the system became starved of assimilable nitrogen, 1 g l^{-1} of sodium propionate containing 1-^{14}C- propionate was added to the fermenter in addition to glucose and fermentation continued for 5 minutes. The cells were then harvested by filtration and the polymer extracted with chloroform. The labelled carbon was found almost exclusively in the chloroform solution indicating that the labelled terminal carbon atom had not been lost as carbon dioxide. Hence at least some propionate had been incorporated into the polymer other than as acetyl CoA.

EXAMPLE 2. (comparative)

Alcaligenes eutrophus mutant NCIB 11599 was grown by aerobic cultivation at pH 6.8 and 34°C in a 5 litre batch fermenter containing 4000 ml f an aqu ous m dium B having the

composition, per litre of deionised water:

$(NH_4)_2SO_4$	4 g
$MgSO_4.7H_2O$	0.8 g
K_2SO_4	0.45 g
H_3PO_4 (1.1 M)	12 ml
$FeSO_4.7H_2O$	15 mg
Trace element solution (as used in Example 1)	24 ml

Glucose was fed to the fermenter at a rate of 8 g hr^{-1}. The amount of assimilable nitrogen in medium B was sufficient to support 26 g of PHB-free cells.

After 40 hours the cells were harvested by centrifugation; they were then freeze dried and the polymer extracted with chloroform.

EXAMPLE 3

Example 2 was repeated except that when the cell weight reached 34 g, propionic acid was fed to the fermenter instead of glucose at a rate of 2.8 g hr^{-1}.

EXAMPLE 4

Example 3 was repeated except that feed of propionic acid was commenced when the cell weight reached 39 g.

EXAMPLE 5

Example 3 was repeated except that the feed of propionic acid was commenced when the cell weight reached 56 g.

EXAMPLE 6

Example 3 was repeated except when the cell weight reached 48 g a single addition of 12 g of propionic acid was made.

EXAMPLE 7

Example 2 was repeated except that medium A was used and propionic acid was fed at a rate of 4 g hr^{-1} instead of glucose throughout the fermentation.

EXAMPLE 8

Example 2 was repeated except that when the cell weight reached 38 g, a mixture of glucose and propionic acid was fed to the ferment r, instead of glucose, at a rate of 5.2 g hr^{-1} glucose and 2.8 g hr^{-1} propionic acid.

EXAMPLE 9

Example 8 was repeated except that the mixture of glucose and propionic acid was fed at a rate of 6.8 g hr^{-1} glucose and 1.2 g hr^{-1} propionic acid, commencing when the cell weight reached 28 g.

In Examples 2 to 9 the propionic acid was added as a solution containing 400 g l^{-1}.

EXAMPLE 10

Example 2 was repeated except that when the cell weight reached 28 g, isobutyric acid was fed to the fermentation vessel at a rate of 2 g hr^{-1} in place of glucose. The isobutyric acid was added as a solution containing 150 g l^{-1}.

In Examples 3 - 6 and 8 - 10 the fermentations were continued until the ratio of the weight of acid fed to the fermenter to the sum of the weight of glucose fed to the fermenter after the cell weight had reached 26 g, i.e. when the system became nitrogen depleted, and the weight of acid fed to the fermenter, reached the values set out in Table 1.

EXAMPLE 11

Example 2 was repeated except that when the cell weight reached 26.4 g, 3-chloropropionic acid was fed to the fermenter instead of glucose at a rate of 4 g hr^{-1} for 5 hours.

EXAMPLE 12

Example 11 was repeated except that feed of 3-chloropropionic acid was commenced when the cell weight reached 34.4 g.

EXAMPLE 13

Example 12 was repeated except that a single addition of 4 g of 3-chloropropionic was made when the cell weight reached 30 g and then glucose was fed at a rate of 6.8 g hr^{-1} for 7 hours.

In Examples 11 - 13 the 3-chloropropionic acid was added as a solution containing 50 g l^{-1}.

EXAMPLE 14

Example 2 was repeated except that when the cell weight reached 31 g, acrylic acid was fed to the fermenter at a rate of 4 g hr^{-1} f r 5 hours instead of glucose. The acrylic

acid was fed as a solution containing 100 g l^{-1}.

Table 1

Example	Acid	Acid feed ratio* (%)	Final cell concentration ($g.l^{-1}$)	Amount of Polymer in cells. (% by weight)
2	none	0	20.0	70
3	propionic	75	15.6	70
4	propionic	50	13.3	60
5	propionic	33	16.0	70
6	propionic	4	13.0	63
7	propionic	100	6.4	55
8	propionic	17	13.6	55
9	propionic	9.5	14.2	67
10	isobutyric	66	13.0	50
11	3-chloropropionic	61	7.4	25
12	3-chloropropionic	33	4.5	20
13	3-chloropropionic	6.5	9.3	35
14	acrylic	50	6.0	25

* acid feed ratio is defined as the weight of acid fed to the fermenter divided by the sum of the weight of glucose added after the cell dry weight reached 26 g and the weight of acid fed to the fermenter.

The amount of comonomer units in the polymers of Examples 2 to 14 was determined (a) by hydrolysis and gas chromatography and (b) by ^{13}C nucl ar magnetic resonance spectrosc py.

The molecular weights of the polymers were determined by gel permeation chromatography.

Chlorine analyses were also performed on the polymers of Examples 2, 11, 12 and 13.

The results are shown in Table 2.

It is seen that little of the chlorine from 3-chloropropionic acid is to be found in the polymer. It would therefore appear that during the metabolism of 3-chloropropionic acid, HCl is lost and the resulting carbon-carbon double bond is hydrogenated and hydrated to give ethyl and 2-hydroxy ethyl substituents as R_1 instead of the expected 2-chloroethyl groups. However the chlorine content of the polymers of Examples 11 - 13 may indicate that some of the chlorine is present as 2-chloroethyl groups.

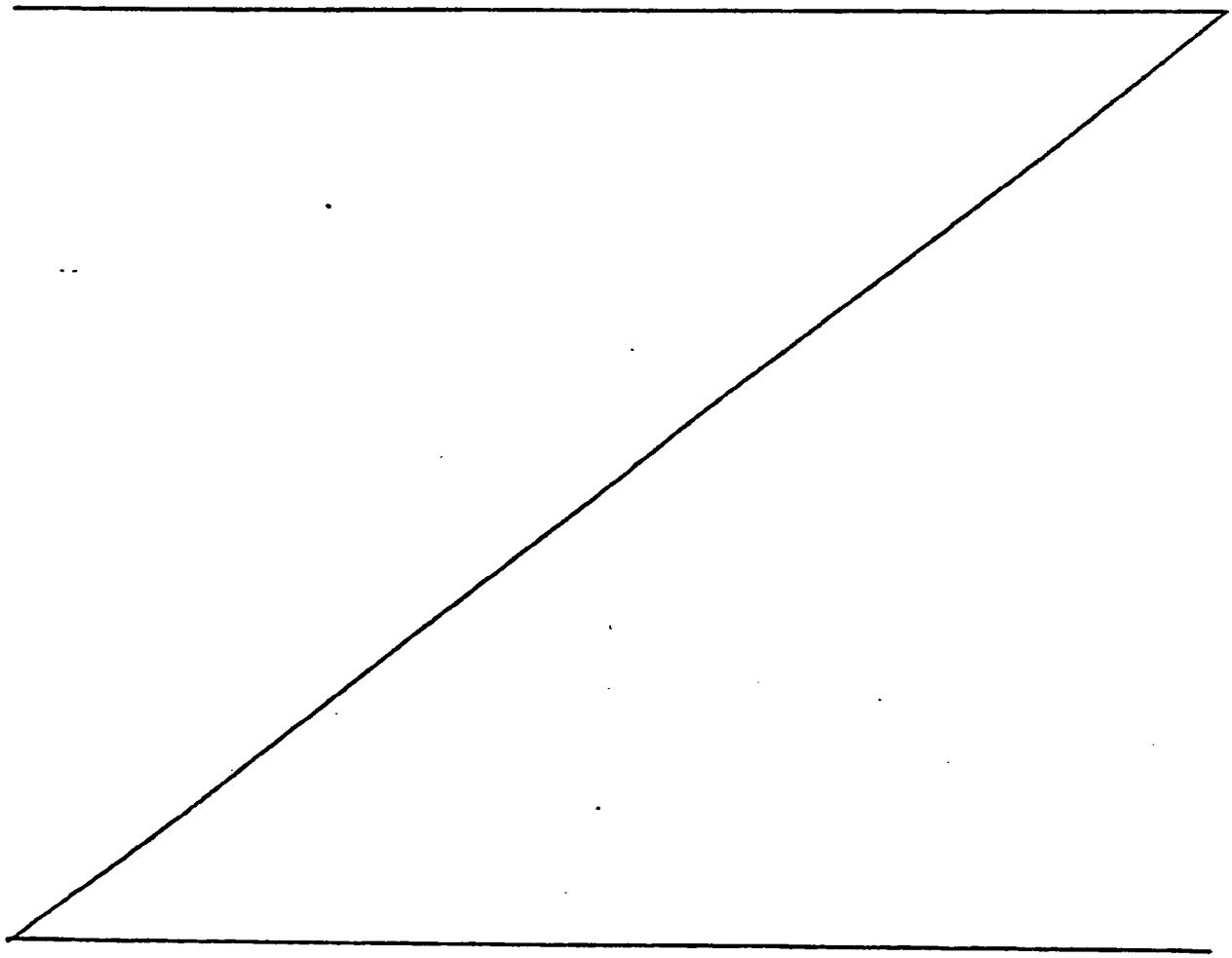
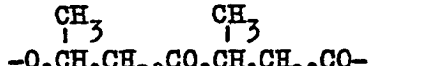


Table 2

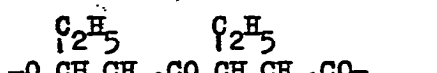
Example	Acid employed	R_1 found	Mole % units II		Molecular weight		chlorine ppm.
			by NMR	by hydrolysis and gas chromatography	Mw x 10^{-3}	Mw/Mn	
2	none	-	0	0	292	2.75	40
3	propionic	ethyl	27	33	207	4.23	
4	propionic	ethyl	24	27	374	1.89	
5	propionic	ethyl	13	14	258	3.50	
6	propionic	ethyl	6	3	348	1.66	
7	propionic	ethyl	25	26	336	1.70	
8	propionic	ethyl	15	14	389	1.67	
9	propionic	ethyl	6	7	243	2.56	
10	isobutyric	ethyl	30	29	274	2.38	
11	3-chloropropionic	ethyl 2-hydroxy ethyl	7 1.8	- -	383	2.99	475
12	3-chloropropionic	ethyl 2-hydroxy ethyl	4 1.2	- -	376	1.77	265
13	3-chloropropionic	ethyl 2-hydroxy ethyl	2 0.6	- -	311	1.99	45
14	acrylic	2-hydroxy ethyl	6.5	-	353	2.36	

High resolution ^{13}C NMR was used to investigate the monomer sequences of the copolymers of Examples 3 - 10. The signal derived from the carbon atom of the carbonyl group was found to occur at different chemical shifts depending upon its environment. Thus with polymers containing the units I and II (where n = 1, $R_1 = C_2H_5$, $R_2 = R_3 = H$) the possible sequences are

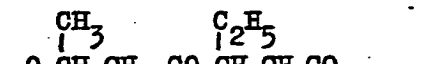
A. butyrate - butyrate, i.e.



B. pentanoate - pentanoate, i.e.



C. butyrate - pentanoate, i.e.



NMR examination of the polymers of Examples 2 - 10 revealed three resonances occurring at 169.07, 169.25 and 169.44 ppm respectively. Following the work of M. Iida et al (Macromoles 11, 1978, p 490) the resonance at 169.07 ppm can be assigned to the butyrate-butyrate sequence A, and that at 169.44 ppm to the pentanoate-pentanoate sequence B. By inference the signal at 169.25 must arise from the butyrate-pentanoate sequence C.

Quantitative analysis of the NMR results of the copolymer of example 10 gave the following results:

sequence A (butyrate-butyrate)	55%
sequence B (pentanoate-pentanoate)	14%
sequence C (butyrate-pentanoate)	31%

These results clearly indicate that the polymer of Example 10 contains a substantial amount of a copolymer of units I and II (where n = 1, $R^1 = C_2H_5$, $R^2 = R^3 = R^4 = H$). However it is possible that some homopolymer of repeat unit I is also present.

All the polymers of Examples 2 - 14 had the D(-) configuration.

The m lting behaviours of the copolymers as extracted was first determined by diff rential scanning calorimetry (DSC)

using the Dupont 1090 system with computerised data analysis. DSC was also performed on the samples after compression moulding at 190°C and slow cooling in the press in order to obtain a fully crystallised product. In each case the specimens were heated at 20°C/min in air and the temperatures at the start (Ts) and peak (Tp) of the melting endotherm, together with its area, were noted. Heating of the annealed sample was continued to 200°C and, after isotherming for one minute to ensure complete melting, it was quenched in liquid nitrogen. The DSC was then re-run in order to determine the glass transition temperature (Tg) of the amorphous phase. Finally the densities of the annealed copolymers were measured by flotation in a density gradient.

The results are shown in Table 3.

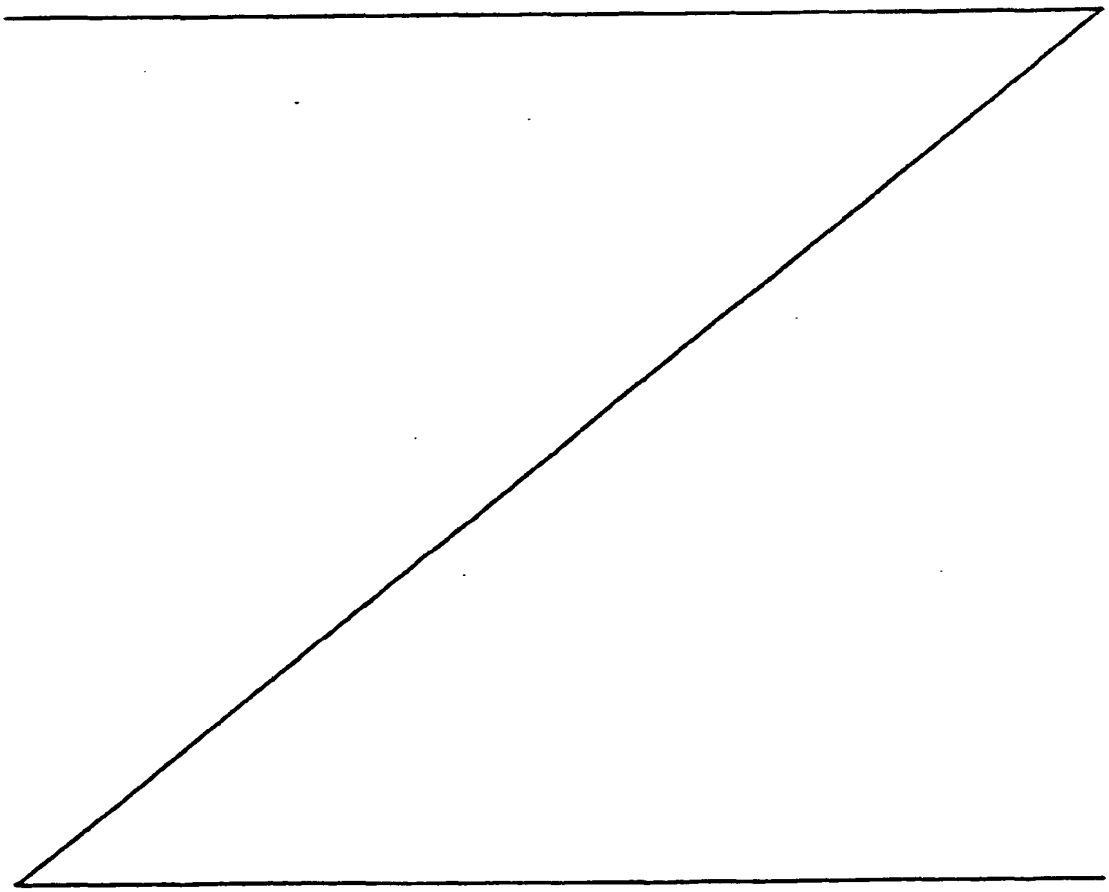


Table 3

Example	DSC on extracted polymer			DSC on annealed polymer				Density g.cm^{-3}
	Ts °C	Tp °C	Area J.g^{-1}	Tg °C	Ts °C	Tp °C	Area J.g^{-1}	
2	140	183	100	5.9	140	191	127	1.256
3	120	125 166	5 20	-1.9	140	171	18	1.172
4	120	170	50	0.8	140	182	44	1.174
5	110	120 170	5 50	2.2	140	177	45	1.200
6	120	172	100	2.7	120	173	96	1.225
7	80	132	34	0.4	80	132	40	1.198
8	110	120 166	6 60	2.0	140	174	43	1.199
9	110	156	89	4.0	110	163	81	1.210
10	50	65 120 168	10 3 25	-2.0	130	172	26	1.138
11	110	170	57	5.0	120	180	73	-
12	110	177	86	4.1	120	173	86	1.182
13	100	172	98	5.9	120	171	96	1.218
14	110	172	84	2.7	110	174	75	1.212

The wide melting ranges of the copolymers indicated that the copolymers were of rather heterogeneous composition. However significant randomisation by ester interchange occurred on annealing since the melting endotherms became much sharper and slightly reduced in area. This is indicative that the polymers are not physical blends of homopolymers but are genuin copolymers.

Multiple DSC peaks were observed for the "as extracted" polymers of Examples 3, 5, 8 and 10.

The area of the melting endotherms gives an indication

of the degree of crystallinity. All the polymers of Examples 3 to 14 after annealing were significantly less crystalline than the control homopolymer of Example 2.

EXAMPLE 15

Alcaligenes eutrophus mutant NCIB 11599 was grown by aerobic cultibation at pH 6.8 and 34°C in a 5 litre batch fermenter containing 4000 ml of an aqueous medium C which was the same as medium B except that the amount of ammonium sulphate was 5.2 g l^{-1} which is sufficient to support 8.5 g l^{-1} of PHB-free cells.

The substrate was glucose supplied at a rate of 5.5 l^{-1} hr^{-1}. When the cell concentration reached 7 g l^{-1}, propionic acid was fed at a rate of 1.58 g l^{-1} hr^{-1} in addition to the glucose. The cells were harvested when the cell dry weight reached 15 g l^{-1}. The cell suspension was spray dried, lipids extracted by refluxing the dried cells with methanol, and the polymer then extracted by refluxing with chloroform. The polymer was recovered by precipitation by adding the chloroform solution to a methanol/water mixture.

The copolymer contained 20 mole % of repeat units II where R^1 was ethyl, R^2, R^3, R^4 were each hydrogen, and n = 1. The copolymer had a molecular weight of 350,000, and was insolubl in cold methyl ethyl ketone. When 2 g of the copolymer was refluxed with 100 ml of methyl ethyl ketone for 1 hour it all dissolved: on cooling the solution a gelatinous mass was formed. In contrast less than 0.1 g of a β-hydroxybutyric acid homopolymer dissolved when 2 g of the homopolymer was refluxed with 100 ml of methyl ethyl ketone. When these solubility tests were repeated with ethanol in place of methyl ethyl ketone, about 0.7 g of the copolymer, and less than 0.04 g of the homopolymer, had dissolved after refluxing for 1 hour.

The solubility of the copolymer in ethanol was also assessed at a lower concentration: thus 0.5 g of the copolymer was refluxed with 1 litre of ethanol for 1 hour. Less than 0.2 g of the copolymer dissolved.

In contrast it is noted that the polymers described by Wallen et al in "Environmental Scienic and Technol gy" 8 (1974)

pages 576 - 579 were said to be soluble in hot ethanol.

EXAMPLE 16

Aqueous media D, E, and F were made up to the following compositions, per litre of deionised water:

Medium D	
$(NH_4)_2SO_4$	12 g
$MgSO_4 \cdot 7H_2O$	1.2 g
K_2SO_4	1.5 g
$CaCl_2$	0.12 g
$FeSO_4 \cdot 7H_2O$	0.1 g
$ZnSO_4 \cdot 7H_2O$	0.006 g
$MnSO_4 \cdot 4H_2O$	0.006 g
$CuSO_4 \cdot 5H_2O$	0.0015 g
H_2SO_4 (concentrated)	1 ml
Medium E	
H_3PO_4 (1.1 M)	2.4 ml
glucose	40 g
Medium F	
H_3PO_4 (1.1 M)	2.4 ml
propionic acid	40 g

A sterilised batch fermenter of nominal capacity 250 litres was filled to the 130 litre mark with a mixture of approximately equal volumes of media D and E. A small sample of the medium in the fermenter was then analysed for nitrogen content. The fermenter was then innoculated with Alcaligenes eutrophus mutant NCIB 11599 and fermentation conducted aerobically at 34°C with automatic pH control at 6.8 by addition of a sodium hydroxide solution.

The amount of assimilable nitrogen present in the fermenter was sufficient to support the growth of the micro-organism to only about 1.2 kg of polymer-free cells. When the cell weight reached about 1.05 kg feed of medium E was commenced at a rate of 6.5 litres/hour.

When the weight of cells reached approximately 1700 g feed of medium E was stopped and feed of medium F commenced at a rate of 6.5 litres/hour, and fermentation continued until about

2.6 kg of cells had been produced.

The cell suspension was then concentrated by centrifugation to a concentration of about 60 g/litre and the polymer extracted therefrom by contacting 1 volume of the suspension with 2 volumes of 1,2-dichloroethane (DCE) in a Silverson mixer at 20°C for 15 minutes. The DCE phase was separated from the aqueous phase, which contained the cell debris, and filtered. The polymer was precipitated by adding 1 volume of the filtered DCE phase to 4 volumes of a methanol/water mixture (4 volumes of methanol to 1 volume of water). The precipitated polymer was collected by filtration, washed with methanol, and dried in an oven for 4 hours at 100°C.

The polymer had a melting range, as determined by differential scanning calorimetry, of about 100°C to 180°C with a peak in the melting endotherm at 168°C.

EXAMPLE 17

The fermentation procedure of Example 16 was repeated except that the changeover from feeding medium E to feeding medium F took place when the weight of cells was approximately 3.5 kg. The medium F was fed at a rate of 11.4 litres/hour for 4 hours and then reduced to 3.2 litres/hour and maintained at this level for a further 9 hours at which stage the weight of cells was about 3.9 kg.

In this example the amount of assimilable nitrogen present in the fermenter was sufficient to support the growth of the micro-organism to only about 1.5 kg of polymer-free cells.

The cell suspension was concentrated by centrifugation and then the polymer was extracted from the concentrated cell suspension by the procedure described in Example 15.

EXAMPLE 18

A 250 litre fermenter was charged and innoculated as in Example 16. The amount of assimilable nitrogen was sufficient to support growth of the micro-organisms only to about 1.9 kg of polymer-free cells. As in Example 16 fermentation was conduct d a robically at 34°C at a pH f 6.8.

When the cell weight reached about 1.0 kg feeds of medium E and of a medium G at rates of 8.7 litres/hour and 4.6 litres/hour respectively were commenced and continued until the cell weight reached about 3.9 kg.

Medium G had the composition, per litre of deionised water:

H_3PO_4 (1.1 M)	1.2 ml
propionic acid	20 g

The cell suspension was concentrated by centrifugation and then the polymer extracted from the concentrated cell suspension by the procedure described in Example 15.

EXAMPLE 19

The procedure of Example 17 was repeated on a larger scale using a fermenter of nominal capacity 1000 litres which was filled to the 500 litre mark with approximately equal volumes of media D and E. In this example the feed of medium E was commenced, at a rate of 25 litres/hour, when the weight of cells was about 4 kg and the feed of medium F was commenced, at a rate of 37.5 litres/hour, when the weight of the cells was about 8 kg. The feeds of media E and F were continued until the weight of cells was about 10 kg. The amount of assimilable nitrogen present was sufficient to support the growth of the micro-organism to only about 4.1 kg of polymer-free cells.

EXAMPLE 20

Example 19 was repeated except that the feed rate of medium F was 25 litres/hour and the fermentation was continued until the weight of the cells was about 11 kg. In this case the amount of assimilable nitrogen present was sufficient to support the growth of the micro-organism to only about 4 kg of polymer-free cells.

The polymers of Examples 16 - 20 were each copolymers containing β-hydroxybutyric acid (HB) units and β-hydroxyvaleric acid (HV) units, and had weight av rage molecular weights above 300,000. They each had the D(-) configuration.

100 parts by weight of ach of the polymers of Examples

16 - 20, and of a β-hydroxybutyric acid homopolymer, were slurried with about 10 parts by weight of chloroform and 1 part by weight of steamic talc, and granulated at room temperature through a domestic mincer. The compositions were then dried to remove the chloroform and extruded at 190°C and regranulated. The resulting granules were injection moulded at 185°C into test bars using a mould temperature of 60°C and a cooling time of 20 sec. The tensile properties were measured according to ASTM D 638-77a at a rate of 50 mm/min and the impact strength assessed by the Izod impact test according to ASTM D 256-78.

The results are shown in Table 4.

Table 4

Example	HV/HB molar ratio		Modulus* (GPa)	Tensile Strength (MPa)	Extension to Break (%)	Izod Impact strength (J/m)	
	by GC	by NMR				1 mm notch	unnotched
16	18/82	20/80	1.47	25	10-31	66	463
17	4/96	6/94	2.98	33	5-7	23	140
18	8/92	7/93	2.10	31	14-19	106	408
19	1/99	4/96	2.70	35	8-14	56	191
20	4/96	4/96	2.48	35	8-15	23	140
homo-polymer	0/100	0/100	3.25	40	6-13	65	115

* at 0.5% extension

EXAMPLE 21

A PVC formulation was made by dry blending the following ingredients at room temperature:

		parts by weight
(i)	vinyl chloride homopolymer (K62)	100
(ii)	a complex tin thiooctyl stabiliser bases on a di-N-dithioglycollic acid ster	1.5

		parts by weight
(iii)	methyl methacrylate/butadiene/ styrene PVC impact modifier	8
(iv)	wax (external lubricant)	0.8
(v)	glyceryl monoester (internal lubricant)	1
(vi)	HB polymer (processing aid)	2

The HB polymer processing aids were

a) a β-hydroxybutyric acid homopolymer prepared by the procedure of Example 2

b) the copolymer of Example 7 (copolymer A)

c) the copolymer of Example 16 (copolymer B)

The processing aids were slurried with about 10% by weight of chloroform, granulated at room temperature through a domestic mincer, dried, melt extruded at 190°C, regranulated, and ground to a particle size below 150 μm before incorporation into the PVC dry blend.

The dry blends were tested as follows:

1. 50 g of the mixture was poured into the mixing head of a Brabender Plastograph maintained at 180°C rotating at 18 rpm under a pressure ram loaded with a 5 kg weight. The time taken for gelation to occur was measured.

2. The mixture was cold compressed to form a candle which was then charged to an extrusion rheometer maintained at 170°C and fitted with a die having a circular orifice of 1 mm diameter and 20 mm land length. After the charge had heated to 170°C, it was extruded at increasing rates. The appearance of the extrudate was noted and the melt extensibility assessed by attempting to draw the extrudate away from the die.

The results are shown in Table 5.

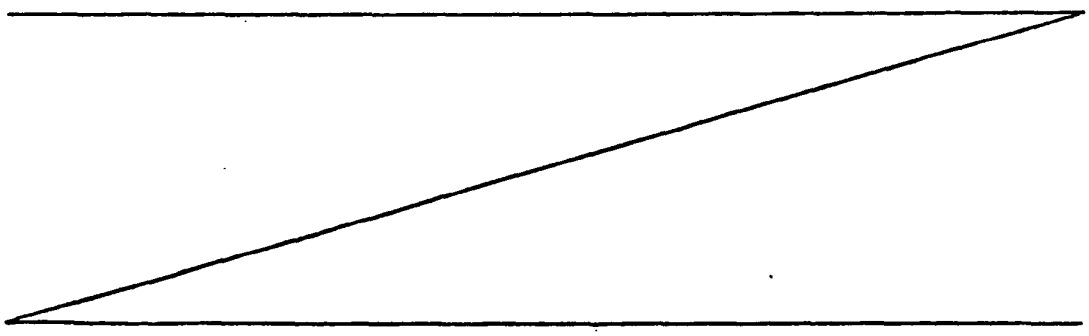


Table 5

Processing aid	Gelation time (min) at 180°C	Extrusion at 170°C	
		Appearance	Melt extensibility
None	12	Severe sharkskin at low extrusion rates; ripple at higher rates	Poor
homopolymer	9.5	Poor with a lot of unmelted polymer clearly visible	Poor
copolymer A	1.0	Excellent - very smooth	Good
copolymer B	1.5	Smooth, but occasional unmelted particles	Fair

This example shows that the copolymers are superior to β-hydroxybutyric acid homopolymer as a vinyl chloride polymer processing aid. The more random copolymer A was clearly superior to the copolymer B..

EXAMPLE 22

A medium H was made up to the following composition:

$(NH_4)_2SO_4$	1 g
KH_2PO_4	2 g
$(Na)_2HPO_4$	3 g
$MgSO_4.7H_2O$	0.2 g
$CaCl_2$	0.01 g
$FeSO_4.7H_2O$	0.005 g
$MnSO_4.4H_2O$	0.002 g
$Na_2CO_3.10H_2O$	0.1 g
$(NH_2)_2$ CO	1.5 g
deionised water	- to 1 litre

This medium had a pH of 7.

Eight 1 litre shake flasks each containing 500 ml of medium H, in which 0.5 g of methacrylic acid had been diss lved, were each innoculated with 5 ml of a starter culture of Nocardia salmonicolor strain ATCC 19149 and incubated at 32°C on a gyratory shaker.

0.5 g of methacrylic acid was added to each flask at intervals of 24, 48 and 72 hours after innoculation, and a final addition of 0.25 g of methacrylic acid was made 96 hours after innoculation. After a total of 108 hours after innoculation the flasks were examined: little or no growth of the microorganism was apparent in any of the flasks. The contents of the flasks were combined and centrifuged to give a pellet of cells which was dried in an oven and weighed. The weight of the pellet was 2.81 g. The cell content of the innoculum was also determined and found to be 69.75 $g.l^{-1}$: Hence the total weight of cells added, as the innoculum, to the flasks was 2.79 g.

It is concluded that, at the concentrations of methacrylic acid employed, this strain does not assimilate methacrylic acid.

1. Copolymers having a weight average molecular weight above 10,000 and containing 99.9 to 50 mole % of repeat units

I $-O.CH(CH_3).CH_2.CO-$

and 0.1 to 50 mole % of repeat units

II $-O.CR^1R^2.(CR^3R^4)_n.CO-$

where n is 0 or 1 and R^1, R^2, R^3, and R^4 are each selected from hydrocarbon radicals; halo- and hydroxy- substituted hydrocarbon radicals; hydroxy radicals; halogen atoms; and hydrogen atoms, provided that, where n is 1, if R^2, R^3, and R^4 are each hydrogen atoms, R^1 is not methyl.

2. Copolymers according to claim 1 wherein n is 1.

3. *Copolymers according to claim 1 or claim 2 wherein* R^1, R^2, R^3 and R^4 each contain less than 4 carbon atoms.

4. Copolymers according to any one of claims 1 to 3 wherein at least one of R^1, R^2, R^3 and R^4 is hydrogen.

5. Copolymers according to claim 4 wherein R^2, R^3 and R^4 are each hydrogen.

6. Copolymers according to any one of claims 1 to 5 wherein R^1 is ethyl.

7. Copolymers according to any one of claims 1 to 6 having a weight average molecular weight above 200,000.

8. Copolymers according to any one of claims 1 to 7 containing 1 to 40 mole % of repeat units II.

9. A process for the production of a thermoplastic polyester wherein a micro-organism that is capable of accumulating a polyester is cultivated in an aqueous medium on a water soluble assimilable carbon containing substrate with at least part of the cultivation being conducted under conditions of limitation of one or more of the essential requirements for microbial growth, but not polyester accumulation, characterised in that, during at least part of the period when the cultivation is so limited, the substrate comprises an organic acid, or salt thereof, that is metabolisable by said micro-organism under said limitation conditions, to a polyester other than one composes solely of $-O.CH(CH_3).CH_2.CO-$ repeat units.

10. A process according to claim 9 wherein the acid is selected from propionic, isobutyric, and acrylic acid.

11. A process according to claim 9 or claim 10 wherein the acid is is the sole substrate for at least part of the period during which the cultivation of the micro-organism is conducted under conditions of limitation of one or more of the essential requirements for microbial growth but not polyester accumulation.

12. A process according to claim 11 wherein the acid is the sole substrate throughout the cultivation of the micro-organism.

13. A process according to any one of claims 9 to 11 wherein the micro-organism is grown using a carbohydrate as a substrate.

14. A process according to claim 13 wherein the carbohydrate is glucose.

15. A process according to claim 13 or claim 14 wherein, for at least part of the period when the cultivation is under conditions of limitation of one or more of the essential requirements for microbial growth, but not polyester accumulation, the substrate is a mixture of the acid and the carbohydrate.

16. A process according to any one of claims 9 to 15 wherein the essential requirement for growth, but not polyester accumulation, that is limited, is the nitrogen source.

European Patent Office

EUROPEAN SEARCH REPORT

Application number
EP 81 30 5186.9

DOCUMENTS CONSIDERED TO BE RELEVANT			CLASSIFICATION OF THE APPLICATION (Int. Cl. ³)
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	
A	GB - A - 1 207 588 (NATIONAL STARCH) * page 1, line 45; formula * —	1	C 08 G 63/06 C 12 P 7/62 // C 08 L 27/06
D,A	ENVIRONMENTAL SCIENCE & TECHNOLOGY Vol. 8, No. 6, June 1974 Washington WALLEN et al. "Poly-β-hydroxyalkanoate from Activated Sludge" pages 576 to 579 * page 578, right-hand column, lines 5 to 9; page 578, left-hand column, lines 28 to 31 * —	1-6	TECHNICAL FIELDS SEARCHED (Int.Cl. ³) C 08 G 63/00 C 12 P 7/00
D,A	US - A - 3 275 610 (COTY) * claim 1 * ——	9	

CATEGORY OF CITED DOCUMENTS

X: particularly relevant if taken alone
Y: particularly relevant if combined with another document of the same category
A: technological background
O: non-written disclosure
P: intermediate document
T: theory or principle underlying the invention
E: earlier patent document, but published on, or after the filing date
D: document cited in the application
L: document cited for other reasons

&: member of the same patent family, corresponding document

X The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
Berlin	12-02-1982	MARX

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